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Neutrophils augment LPS-mediated pro-inflammatory signaling in human lung epithelial cells

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ABSTRACT

Background: The role of polymorphonuclear neutrophils in pulmonary host defense is well recognized. The influence of a pre-existing inflammation driven by neutrophils (neutrophilic inflammation) on the airway epithelial response toward pro-inflammatory exogenous triggers, however, is still poorly addressed. Therefore, the aim of the present study is to investigate the effect of neutrophils on lipopolysaccharide (LPS)-induced pro-inflammatory signaling in lung epithelial cells. Additionally, underlying signaling pathways are examined.
Methods: Human bronchial epithelial cells (BEAS-2B) were co-incubated with human peripheral blood neutrophils or bone-marrow derived neutrophils from either C57BL/6J wild type or nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase deficient ($p47^{phox-/-}$) mice. Upon stimulation with LPS, interleukin (IL)-8 production and reactive oxygen species (ROS) generation were measured. Additionally, activation of the extracellular signal-regulated kinases (ERK) 1/2 and nuclear factor (NF)-κB signaling pathways was analyzed.
Results: Our studies show that the presence of neutrophils synergistically increases LPS-induced IL-8 and ROS production by BEAS-2B cells without inducing cytotoxicity. The observed IL-8 response to endotoxin increases in proportion to time, LPS-concentration and the number of neutrophils present. Moreover, this synergistic IL-8 production strongly correlated with the chemotactic properties of the co-incubations and significantly depended on a functional neutrophilic NADPH oxidase. The presence of neutrophils also augments LPS-induced phosphorylation of ERK1/2 and IκBα as well as NF-κB RelA DNA binding activity in BEAS-2B cells.
Conclusions: Our results indicate that the pro-inflammatory effects of LPS toward lung epithelial cells are amplified during a pre-existing neutrophilic inflammation. These findings support the concept that patients suffering from pulmonary neutrophilic inflammation are more susceptible toward exogenous pro-inflammatory triggers.

1. Introduction

Although breathing is mandatory for all aerobic life forms, it also implicates constant exposure of the respiratory tract to noxious environmental stimuli and airborne pathogens. As the front line of pulmonary defense against these potential threats, the airway epithelial cells are not only providing a physical barrier but will also enhance the clearance of these invading micro-organisms by stimulating the expression of various inflammatory mediators including mucus proteins and cytokines [1,2]. In general, NOX enzymes can produce large amounts of ROS, mainly superoxide anion radical and H_2O_2, upon stimulation with various bacterial and environmental triggers including endotoxins and ambient particulate matter [2]. Classically, the phagocytic NOX has been regarded as the main player of systemic innate immune responses [3]. Upon intrusion of microbial pathogens, neutrophils will be recruited from the circulation to attack and neutralize these pathogens by producing ROS via their NOX-driven respiratory burst. Alternatively, it has become appreciated that the lung also comprises other NOXs capable of producing ROS. Indeed, it is shown that the NADPH oxidase homologs DUOX1/2 are the primary sources of pulmonary epithelial production of H_2O_2 [4,5]. Being a substrate for lactoperoxidase and

Abbreviations: BEAS-2B, human bronchial epithelial cultured cell line; CPH, 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine; DUOX1/2, dual oxidases 1 and 2; EPR, electron paramagnetic resonance; ERK1/2, extracellular signal-regulated kinases 1/2; H_2O_2, hydrogen peroxide; IκBα, inhibitory part of NF-κB; IL, interleukin; KO, $p47^{phox-/-}$ knockout; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NOX, NADPH oxidase; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; TLR, toll-like receptor; WT, wild type

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myeloperoxidase, H_2O_2 contributes to the formation of respectively hypothiocyanous acid and hypochlorous acid, which both possess strong bactericidal capacities [6–9]. Other NOX enzymes displaying pulmonary expression include NOX2 and, to a lesser extent, NOX4, although the expression of these enzymes largely resides in non-epithelial cells present in the lung including respectively phagocytic inflammatory cells (neutrophils and macrophages) and fibroblasts [2,4,10,11]. Interestingly, it has recently been suggested that ROS produced by NOX4 play a role in tissue repair functions and pulmonary fibrogenesis as this homolog is upregulated in lungs of mice subjected to noninfectious injury as well as in lungs of pulmonary fibrosis patients [12].

An important player in the respiratory epithelial defense, which is also highly ROS-inducible, is the chemokine IL-8/CXCL8. Although IL-8 displays various inflammatory effects, including induction of ROS production and modulation of histamine release, its key activity is the chemotaxis of PMN toward infectious sites [13,14]. Expression of the IL-8 gene is regulated by several pathways, as can be deducted from the presence of binding sequences for various transcription factors, including NF-κB and AP-1, on its promoter region [15,16]. The transcription factor AP-1 is composed of members of the jun and fos DNA-binding protein families [15,17]. The abundance and activity of AP-1 relies on the phosphorylation of both the jun and fos proteins [18] by respectively c-Jun NH_2-terminal kinases and ERKs. Upon activation, ERKs translocate into the nucleus and phosphorylate fos proteins. For c-fos, fosB and fra1 it has already been shown that this phosphorylation occurs on serine and/or threonine residues in the COOH-terminal domain [19]. The transcription factor NF-κB is present in the cytoplasm as an inactive complex, typically existing as a dimer of the RelA and p50 subunits that is bound to the inhibitory protein IκB [20,21]. The classical NF-κB activation involves phosphorylation of the cytosolic inhibitor protein IκBα at the serine residues 32 and 36 by the IKK enzyme complex, leading to the protein's rapid ubiquitination at lysines 21 and 22 and subsequent annihilation by the 26S proteosome [20]. Consequently, the dissociated NF-κB dimer is released and translocates to the nucleus.

Upon secretion of IL-8 by the airway epithelium, large amounts of PMN will migrate into the lung lumen where they become activated and undergo a respiratory burst [22]. This burst, initiated by NOX2, is a self-destructing process characterized by an extensive ROS production. In resting cells, NOX2 is a fully inactive enzyme that exists as a cytosolic complex, containing $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ [3,23]. On activation, its regulatory subunit $p47^{phox}$ becomes phosphorylated and translocates to the membrane where it will bind to its transmembrane cytochrome unit comprising both NOX2 and the closely associated $p22^{phox}$ [2,3,23]. The distinct movement of GTP-binding protein Rac to the membrane enables the combination of all required regulatory units to form an active enzyme, capable of transferring electrons from NADPH via a bound flavin adenine nucleotide to molecular oxygen to form superoxide anion radical [3,23,24].

Due to their biological activity, stimulated neutrophils may represent a double-edged sword for pulmonary homeostasis. On the one hand, the appropriate recruitment of neutrophils toward the infected part of the airway results in killing the invading microorganisms and thus preserving the homeostasis. On the other hand, those same activated neutrophils also possess the ability to seriously damage host tissue. For example, activated neutrophils have been shown to induce oxidative DNA-lesions [25,26] and to inhibit DNA nucleotide excision repair [27] in pulmonary epithelial cells. Moreover, excessive neutrophil influx and/or incomplete apoptosis of activated neutrophils may result in disproportionate ROS production and prolonged inflammation. Indeed, neutrophil dysregulation has been associated with various pulmonary inflammatory diseases including asthma, adult respiratory distress syndrome and chronic obstructive pulmonary disease [28,29]. However, the influence of a pre-existing pool of neutrophils, resulting from either a yet-to-be resolved inflammation or a chronic lung condition, on the airway epithelial response toward noxious stimuli is not known. Therefore, the aim of the current study is to investigate the effect of neutrophils on LPS-induced pro-inflammatory signaling in lung epithelial cells.

2. Materials and methods

2.1. Chemicals

LPS from *Escherichia coli* (0.26:B6), PMA, transferrin, insulin, dexamethasone, bovine pituitary extract, trypsin, 4-aminobenzoic acid hydrazide (4-ABAH) and luminol (3-aminophthalhydrazide) were purchased from Sigma (St. Louis, MO). Hoechst 3342, penicillin, streptomycin, HBSS, DMEM/Ham's F12 (1:1), trizol and BSA were obtained from Gibco Invitrogen (Paisley, UK). Epidermal growth factor was acquired from Biomol (Hamburg, Germany) while CPH was purchased from L-Optik (Berlin, Germany). The RNeasy mini kit was provided by Qiagen (Germany). The iScript cDNA Synthesis kit and SYBR© Green Supermix were supplied by Biorad (Germany). TLR-4, IL-8 and β-actin primers were ordered at Eurofins MWG Operon (Germany) with the following sequences: β-actin forward: 5′-CCC CAG GCA CCA GGG CGT GAT-3′; β-actin reverse: 5′-GGT CAT CTT CTC GCG GTT GGC CTT GGG GT-3′; TLR-4 forward 5′-TGC ATG GAG CTG AAT TTC TAC-3′; TLR-4 reverse: 5′-TGA GCC ACA TTA AGT TCT TTC-3′. Cholera toxin was generously provided by Dr J. de Jonge (Maastricht University, the Netherlands). All other chemicals were of analytical grade.

2.2. Cytotoxicity

Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay as a marker of plasma membrane damage using a colorimetric kit (Roche, Switzerland) according to the manufacturer's instructions.

2.3. Isolation of human and murine PMN

The fresh isolation of human PMN, using blood of 3 healthy male and 3 healthy female volunteers (ages 30–45), was performed as described previously with minor modifications [26]. In short, blood was diluted with cold HBSS (1:1) after which gradient centrifugation was used to remove the lymphocytes. The remaining lowest layer containing PMN and erythrocytes was then suspended in cold lysis buffer to lyse the erythrocytes. The remaining pellet containing PMN only was suspended in HBSS and counted using a Bürker chamber. Viability was tested using Trypan Blue dye exclusion (0.4%). Keeping all solutions and PMN on ice all the time to prevent premature activation, this isolation method consistently yielded PMN with a viability >95%.

For the fresh isolation of murine PMN, the femurs and tibias of specific pathogen-free C57BL/6J (WT) and $p47^{phox-/-}$ knockout (KO) mice were used [30]. Animals were obtained from Taconic (Lille Skensved, Denmark) and had ad libitum access to food and water containing antibiotics (800 mg sulfamethoxazol and 160 mg trimethoprim per liter). Upon sacrificing the animals using a phentobarbital overdose, bone-marrow derived PMN were obtained according to the method originally described by Boxio et al. [31]. Upon harvesting the PMN fraction using a three-layer Percoll gradient, the similar procedure as for the human PMN was used with the exception that HBSS was replaced by HBSS containing 0.5% BSA. Consequently, this isolation method, combined with cooling all the solutions and PMN again, also resulted in a constant and viable (>95%) yield. No remarkable differences regarding reactivity were observed between PMN isolated from male and female volunteers.

2.4. Cell culture and experimental procedures

The immortalized human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection and cultured in DMEM/Ham's F1 medium (1:1) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (10 ng/ml), dexamethasone (0.1 μM), cholera toxin (10 ng/ml), bovine pituitary extract (15 μg/ml) and BSA (0.5 mg/ml) at 37 °C and 5% CO_2. For the various experiments, cells were grown to confluence in either 60 mm dishes (signaling and RT-PCR), 24 well plates (cytotoxicity and IL-8 production) or 96 well plates (chemiluminescence) and starved for 24 h by reducing the concentrations of supplement to 10%. Subsequently, cells received fresh starvation medium for another hour after which they were stimulated with LPS (10 or 100 μg/ml). When indicated, cells were (pre-)treated with the appropriate inhibitors. For the general co-incubations, BEAS-2B cells were incubated in the presence or absence of an equal PMN number, but in specific experiments 3 times lower and higher PMN numbers were also applied. When indicated, PMN inactivated by heat (46 °C for 7.5 min) were used. After co-incubation, PMN were washed off using cold HBSS as described previously [26]. Cells or conditioned media were collected as indicated in the Results section.

2.5. Measurement of IL-8 protein levels

IL-8 protein levels in the conditioned media were quantified after 4 and 24 h of stimulation using an IL-8 ELISA kit (CLB/Sanquin, The Netherlands) based on appropriate and validated sets of monoclonal antibodies. The assay was performed as described in the manufacturer's instructions.

2.6. Chemotaxis

Upon fresh isolation as described above, human PMN were stained with Hoechst 3342 (12 μg/ml) for 30 min at 37 °C followed by two washing steps with cold HBSS. To measure the chemotactic properties of the conditioned media after 24 hour stimulation with LPS, the samples were transferred to the lower wells of a 96-transwell plate (Costar, pore size 3 μm). Next, the stained PMN were carefully added to the upper wells of the plate in a final concentration of 300,000 cells/ml. Following 90 minute incubation at 37 °C, the migration of the PMN to the lower wells was measured using fluorescence (excitation wavelength 350 nm, emission wavelength 460 nm).

2.7. Western blot analysis

To examine the phosphorylation of ERK1/2 and IκBα, cells were stimulated with LPS for 15 min, washed twice with cold PBS and collected in 100 μl lysis buffer (1% NO-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing freshly added protease inhibitor cocktail (Roche, Prenzberg, Germany; 1 tablet/12 ml buffer). After incubation for 15 min on ice, cell lysates were collected by scraping and centrifuging (16,000 *g*, 5 min, 4 °C) to remove cell debris. Protein concentrations were determined by BioRad-Assay (according to the Bradford method) and samples were analyzed by Western blotting. To this extent, samples were separated at equal protein concentrations (10–15 g) by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes. Membranes were blocked to prevent non-specific protein binding and incubated (24 h, 4 °C) with specific polyclonal antibodies against phosphorylated ERK1/2 (1:1000), total ERK1/2 (1:1000), phosphorylated IκBα (1:2000) or tubulin (1:5000). Visualization occurred using a secondary anti-mouse igG whole protein horseradish-conjugated antibody (1:1000 for phosphorylated and total ERK/12; 1:2000 for phosphorylated IκBα and 1:5000 for tubulin) and enhanced chemiluminescence (Pierce). Quantification was performed using the software Quantity One (Bio-Rad).

2.8. NFκB RelA binding activity assay

To investigate the DNA binding activity of the NFκB RelA subunit, an ELISA based DNA binding activity assay was performed using the nuclear extracts of the BEAS-2B cells from the various incubations. Nuclear fractions were prepared with a commercially available nuclear extraction kit (Active Motif, Belgium). The assay was performed using 20 μg of nuclear extract according to the manufacturer's recommendations. As a positive control, 2.5 μg Jurkat cell nuclear extract was used as supplied in the kit (cat no. 36014). Absorbance was read at 450 nm, while 650 nm was used as the reference wavelength.

2.9. ROS formation measurements by luminescence and EPR

For the measurement of ROS by luminol-enhanced chemiluminescence, BEAS-2B cells were seeded in a white Maxisorp 96-well plate (Nunc, Germany) and grown to confluence. Upon starvation and washing the cells twice with warm PBS, the cells were stimulated with LPS (100 μg/ml) or the known NOX2 activator PMA in the presence or absence of PMN. Next, the H_2O_2-sensitive probe luminol ($5*10^{-4}$ M) was added and chemiluminescence was recorded for 60 min at 37 °C using a luminometer (Multi-Bioluminat, Berthold, Germany).

ROS production by murine PMN was analyzed with EPR spectroscopy using the spin probe CPH that is highly sensitive for $O_2^{\bullet -}$. PMN were suspended in cold HBSS-BSA ($5*10^5$ cells/ml) and, upon addition of CPH (0.5 mM), stimulated with PMA (50 ng/ml) or LPS (10 μg/ml) for 120 min at 37 °C. ROS generation was evaluated using a MiniScope MS200 Spectrometer (Magnettech, Berlin, Germany) with the following instrumental settings: RT; magnetic field 3360 G; sweep width 100 G; scan time 60 s; number of scans 1 and modulation amplitude 1800 G. Data shown include a representative example out of three independent experiments, comprising the peak triplet characteristic for CPH.

2.10. Quantitative RT-PCR

To confirm the translation of TLR4 in the cells used, confluent BEAS-2B layers were treated with LPS for 24 h, scraped and homogenized in Trizol. RNA was extracted using the RNeasy mini kit according to the instructions provided by the manufacturer. The quality and quantity of the obtained RNA were assessed using spectrophotometrical analysis at wave lengths of 230, 260, 280, and 320 nm. For isolated RNA, a ratio of at least 1.7 for values obtained at 280 nm and 260 nm was considered acceptable.

Next, a 0.5 μg cDNA sample was synthesized using the iScript cDNA Synthesis kit. To this end, all cDNA samples were diluted 15 times in RNase-free water prior to qRT-PCR. Briefly, 2.5 μl of 0.3 μM forward and 2.5 μl 0.3 μM reverse primer was added to 2.5 μl water, 5 μl diluted cDNA and 12.5 μl SYBR© Green Supermix (Biorad) in each well of a 96 well plate to generate 25 μl of reaction mixture. A MyiQ Single Color Real Time PCR Detection System (BioRad) was used to perform qRT-PCR. The denaturation step, that took place at 95 °C for 3 min, was followed by 40 cycles at 95 °C (15 s) and 60 °C (45 s). An additional step involving the generation of a melt curve (60–95 °C) was performed to ensure that the correct product was amplified and quantified. The results were analyzed using the MyiQ Software system (BioRad) and expressed according to the 2-ΔΔCt method as relative gene expressions (fold increase) of representative control samples, normalized for the housekeeping gene β-actin.

2.11. Data expression and statistical analysis

With exception of the signaling and RT-PCR experiments that were performed in single dishes of three independent experiments, all incubations were performed in duplicate wells in each of at least three independent experiments. Data are expressed as median, range and inter-quartile ranges unless otherwise mentioned.

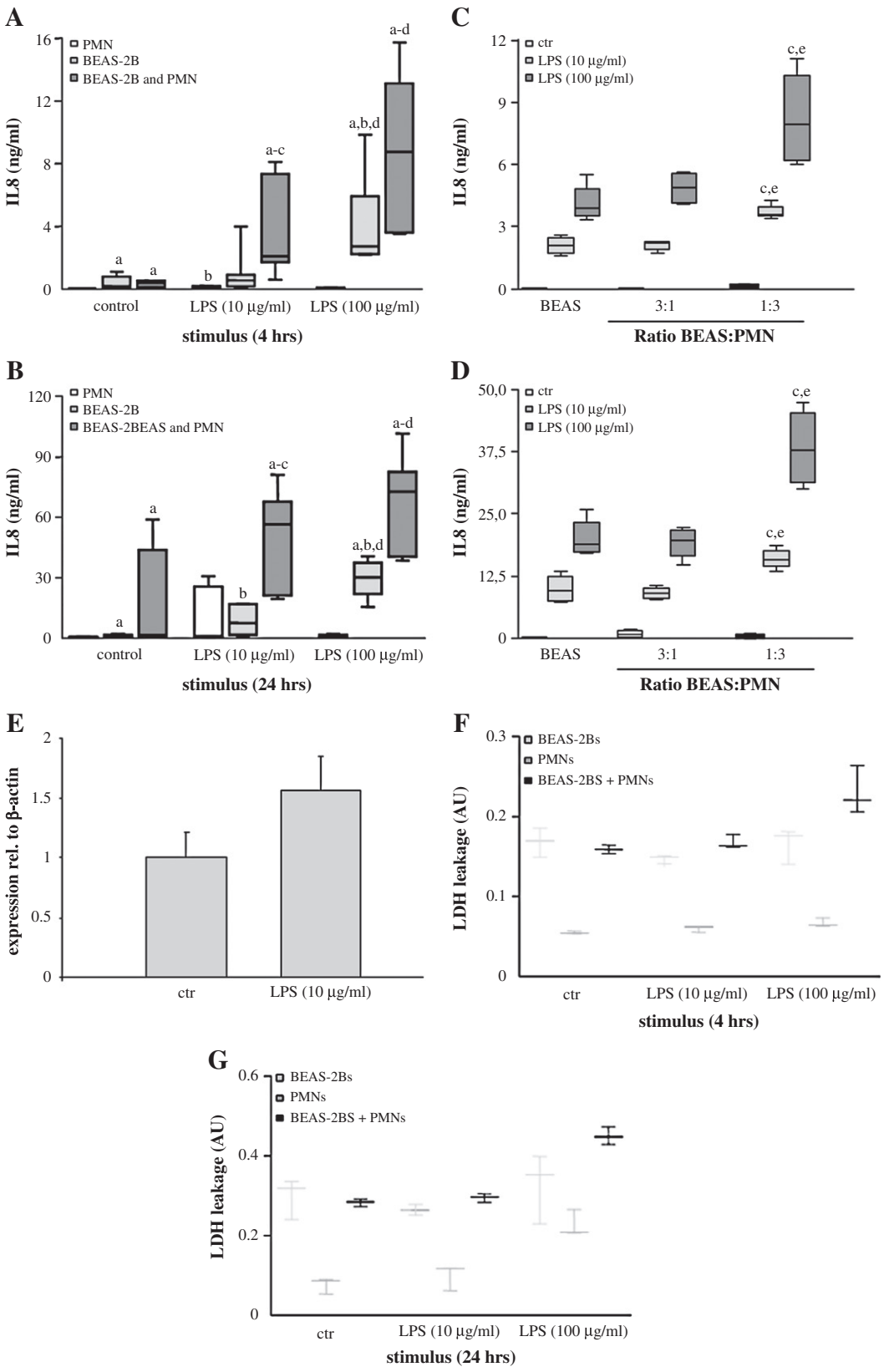

Fig. 1. LPS dose-dependently induces IL-8 production in human PMN, BEAS-2B and the combination of both cell-types. Upon starvation, BEAS-2B cell monolayers were stimulated with LPS (10 or 100 µg/ml) for either 4 (panels A and C) or 24 (panels B and D) hours in the absence or presence of various numbers of freshly-isolated human PMN. Afterward, the IL-8 secretion in the medium was measured. Additionally, the IL-8 production by LPS-stimulated PMN in the absence of BEAS-2B cells was determined. Moreover, the constitutive and LPS-induced gene expressions of TLR4 was analyzed after 24 h (panel E). Finally, cytotoxicity of the different LPS treatments on BEAS-2B cells was evaluated for 4 (panel F) and 24 h (panel G). Except for panel E, data are expressed as median, range and inter-quartile ranges of either n = 6 with 3 different PMN donors (panels A, B, F, G and H) or n = 4 with 2 different PMN donors (panels C and D). Data in panel E are expressed as average ± S.E.M. of n = 3. a = $P<0.05$ compared to the corresponding incubation with PMN only; b = $P<0.05$ compared to the corresponding incubation without LPS; c = $P<0.05$ compared to the corresponding incubation with BEAS-2B; d = $P<0.05$ compared to the corresponding incubation with 10 µg/ml LPS; and e = $P<0.05$ compared to the corresponding incubation with ratio 3:1.

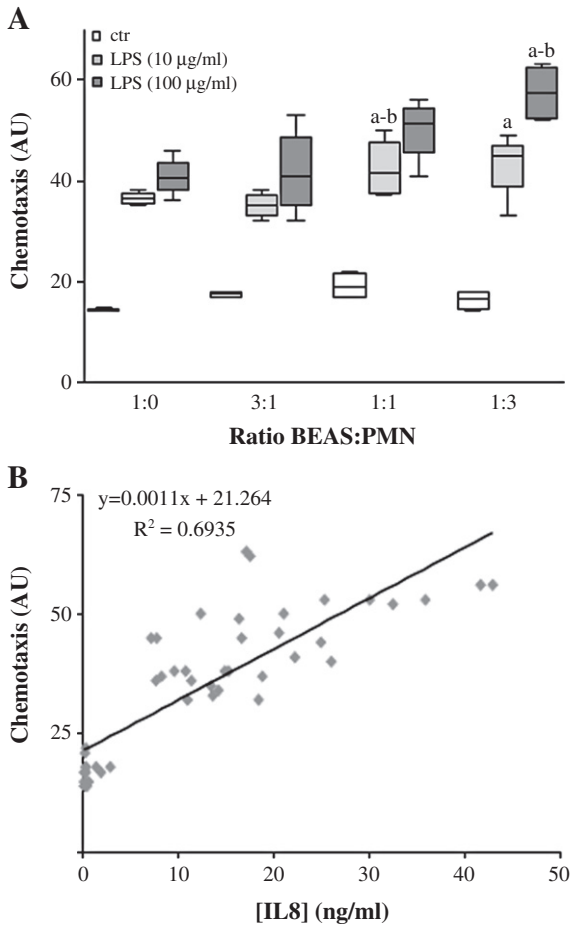

Fig. 2. LPS dose-dependently provokes the release of chemo-attractants by BEAS-2B cells in relation to both the number of PMN present and the IL-8 produced. Upon 4 (panel A) and 24 (panel B) hour stimulation with LPS (10 or 100 µg/ml), the supernatants of BEAS-2B cells co-incubated with various numbers of PMN were added to the lower wells of 96-transwell plate (pore size of 3 µm). Freshly-isolated human PMN were stained with Hoechst 33342 (12 µg/ml) and then added to the upper wells of the plate in a final concentration of 300,000 cells/well. Following 90 minute incubation at 37 °C, the migration of the PMN to the lower wells was measured using fluorescence (excitation wavelength 350 nm, emission wavelength 460 nm). Data are expressed as median, range and inter-quartile ranges for all co-incubations (panels A and B; n = 4 with 2 different PMN donors) and correlated with the IL-8 production for the co-incubation with equal amounts of BEAS-2B cells and PMN (panel C). a = $P<0.05$ compared to the corresponding incubation with ratio 1:0 and b = $P<0.05$ compared to the corresponding incubation with ratio 3:1.

Statistical analysis was performed using the non-parametric Mann–Whitney *U* test (SPSS 15.0). Differences were considered significant if $P<0.05$.

3. Results

3.1. LPS induces synergistic IL-8 production and chemoattractant release in a co-incubation of BEAS-2B cells and PMN

As shown in Fig. 1A and B, LPS induced increased IL-8 production in human BEAS-2B epithelial cells and PMN after 4 and 24 h, respectively. Only the highest LPS concentration did not induce any IL-8 production in the PMN alone after 24 h. This finding is probably due to toxicity, leading to quick and complete bursting, as a result of the long exposure to such a high dose (Fig. 1G). In the BEAS-2B cells, this LPS effect appeared to be clearly dose-dependent at both time-points. Our findings are in line with previous studies showing that both cell types will produce IL-8 upon stimulation with the TLR-4 ligand LPS [14,32–34]. Indeed, in accordance with other studies [32,35,36], our RT-PCR studies confirmed the translation of this receptor in the BEAS-2B cells (Fig. 1E). Moreover, co-incubating the epithelial cells with human PMN did not result in any additional cytotoxicity in the BEAS-2B (Fig. 1F and G).

Next, the effect of LPS on the IL-8 production by a co-incubation of both BEAS-2B cells and PMN in a ratio of 1:1 was examined. In co-incubations of both cell types in a 1:1 ratio, LPS caused an IL-8 production that was far higher than the sum of these cytokine levels produced by the two separate cell types (Fig. 1A and B). Again, this LPS-effect was significantly dose-dependent at both time-points. Moreover, after 24 h without any external stimulus, this co-incubation also resulted in an enhanced IL-8 production compared to the corresponding incubation with only PMN (Fig. 1B).

To explore the influence of PMN number on this observed synergistic IL-8 production, the co-incubations have also been performed in a 3:1 and 1:3 ratio. As depicted in Fig. 1, the LPS-induced IL-8 production by BEAS-2B cells increases proportionally to the number of PMN present after both 4 (panel C) and 24 (panel D) hours.

Based on the knowledge that IL-8 is an effective chemotactic factor for neutrophils [37], the migration of human neutrophils toward supernatants of BEAS-2B cells treated with LPS in the absence or presence of an equal number of PMN has been examined. Fig. 2A and B show that the supernatants of LPS-treated BEAS-2B cells provoked significant migration of PMN in a dose-dependent manner. Interestingly, in analogy with the IL-8 production, this chemotactic effect of the BEAS-2B supernatants was dependent on time, LPS concentration and the number of neutrophils present. Moreover, the chemotaxis induced by the supernatants of BEAS-2B cells treated in the presence of an equal number of PMN displayed a strong and significant correlation with the corresponding IL-8 production of these co-incubations (Fig. 2C).

3.2. The presence of human PMN augments the LPS-induced phosphorylation of ERK1/2 and IκBα as well as the NFκB RelA DNA binding activity in BEAS-2B cells

It has previously been demonstrated that LPS-induced pulmonary IL-8 production is associated with increased activation of ERK1/2 and NF-κB [1,14,38,39]. Consequently, the involvement of both signaling molecules in the synergistic LPS-induced IL-8 production observed in our co-incubation was examined.

Western blot analysis demonstrated a significant activation of ERK1/2 by both LPS concentrations (Fig. 3A). The presence of an equal number of human PMN further enhanced this LPS-induced ERK1/2 activation, although this enhancement was only statistically significant when cells were stimulated with the lower LPS concentration of 10 µg/ml. Similarly, both LPS concentrations significantly induced the phosphorylation of the NF-κB inhibitory part IκBα, a process that leads to the activation of this transcription factor (Fig. 3B). Indeed, LPS was also capable of significantly increasing the DNA binding activity of the NF-κB RelA subunit (Fig. 3C). In accordance with the ERK1/2 findings, the IκBα phosphorylation was also more pronounced when the BEAS-2B cells were stimulated in the presence of PMN, although statistical significance of this effect was only observed when 10 µg/ml LPS was used. Additionally, the LPS-induced DNA binding activity of the NF-κB RelA subunit was significantly increased in the presence of PMN when compared to the control incubation without LPS. Interestingly, just the presence of PMN without additional stimulation with LPS also induced a significant phosphorylation of both transcription factors as well as an increased DNA binding activity of NF-κB RelA in the BEAS-2B. This finding is in accordance with the observation that PMN without additional stimulation already display a minor burst in the control experiments due to the incubation temperature (37 °C).

Additionally, we have monitored the activity of human PMN over time to check whether these cells could also affect the ERK1/2 and

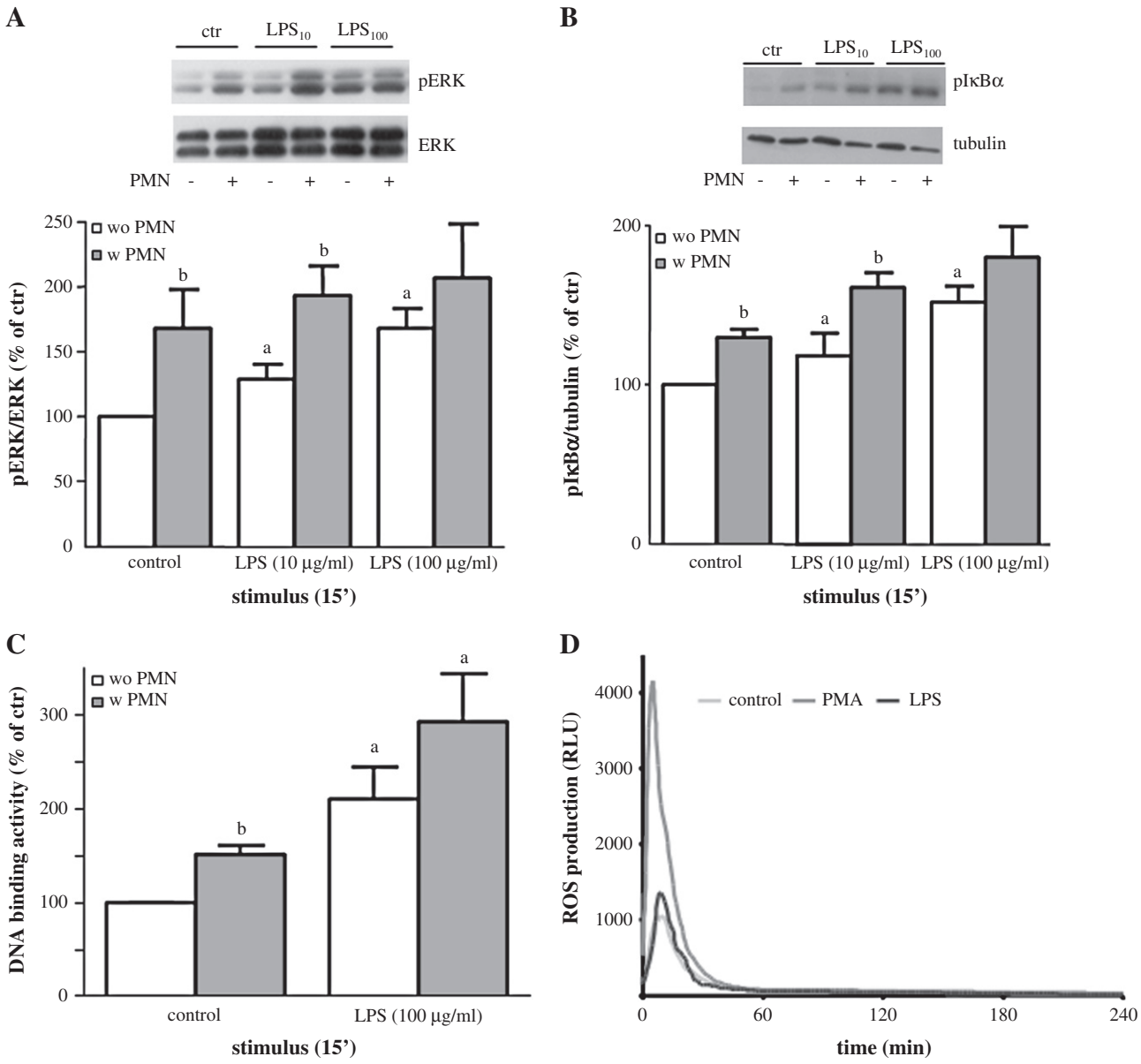

Fig. 3. LPS-induced ERK1/2 and IκBα phosphorylation and DNA binding activity of NF-κB RelA are more distinct in the presence of human PMN. Starved BEAS-2B monolayers were stimulated for 15 min with LPS (10 or 100 µg/ml) and harvested for Western blot analysis of phosphorylated and total ERK1/2 (panel A) or phosphorylated IκBα and tubulin (panel B) or processed to obtain nuclear extracts for analyzing NF-κB RelA DNA binding activity (panel C). Where indicated, cells were incubated in the presence of freshly-isolated human PMN (1:1 ratio) which were washed off before harvesting as described in the Materials and methods section. Additionally, the activity (panel D) of human PMN was monitored over time to check whether the effects of these cells on ERK1/2 and IκBα phosphorylation should also be analyzed at later time points. Representative blot and quantitative analyses by densitometry (panels A and B, mean ± S.E.M. of n = 3 with different PMN donors) or mean ± S.E.M. (panels C and D n = 3 with different PMN donors) are shown. a = $P<0.05$ compared to the corresponding incubation without LPS. b = $P<0.05$ compared to the corresponding incubation without PMN.

NF-κB signaling cascades at a later time point. As can be deducted from Fig. 3D, the activity of human PMN was completely and permanently abolished after 30 min. Consequently, it can be stated that no second burst of activation will occur within our in vitro co-incubation system.

3.3. LPS-mediated ROS production is synergistically increased in co-incubations of BEAS-2B and human PMN

Upon showing ERK1/2 and NF-κB activation in the observed synergistic IL-8 production by the co-incubation, the possible role of ROS herein was assessed using luminol-enhanced chemiluminescence. In contrast to PMN, BEAS-2B cells alone did not produce significant amounts of ROS upon treatment with LPS. In the co-incubations, LPS induced an ROS production that by far exceeded the sum of the ROS produced by the two cell types separately (Fig. 4).

3.4. Neutrophilic NADPH-oxidase plays a crucial role in ROS production by the co-incubation of BEAS-2B and PMN

In view of the observed synergistic ROS production in the co-incubations, we evaluated the role of the neutrophilic NADPH oxidase complex herein using two independent approaches. First, the role of NADPH oxidase in human neutrophils was assessed by using heat inactivation to temporarily inactivate enzyme function. Second, we replaced the human neutrophils by neutrophils isolated from bone marrow of

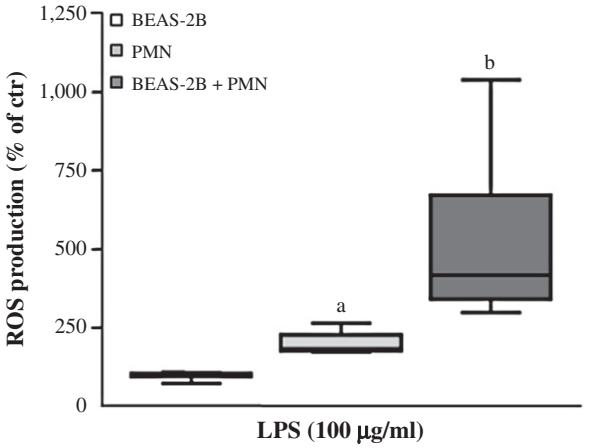

Fig. 4. Stimulating a PMN-BEAS-2B (1:1) co-incubation with LPS results in a synergistic ROS production. Cumulative ROS-production by starved BEAS-2B cells treated with LPS (100 µg/ml) in the absence or presence of freshly-isolated PMN (1:1 ratio) was measured during 60 min using luminol-enhanced chemiluminescence. Additionally, the ROS-production by LPS-stimulated PMN alone was also determined. Data are shown as median, range and inter-quartile ranges of n = 6 with 3 different PMN donors. a = $P<0.05$ compared to the corresponding incubation in only BEAS-2B cells and b = $P<0.05$ compared to the corresponding incubation in only BEAS-2B cells and only PMN.

WT mice or p47$^{phox-/-}$ KO mice lacking a functional NOX2 complex [30]. Using either approach, a significantly reduced ROS production by PMN upon stimulation with LPS or the positive control, the known NOX2 activator PMA, was observed (Fig. 5A and 6). Next, the role of the neutrophil NADPH oxidase on the synergistic ROS production observed in the co-incubation of BEAS-2B cells and PMN was evaluated by treating BEAS-2B cells with LPS (Fig. 5B) in the presence of normal or heat-inactivated human PMN. In accordance with our expectations, the synergistic ROS production previously observed when both cell-types were simultaneously stimulated with LPS was significantly annulled when the PMN were first heat inactivated.

3.5. Synergistic IL-8 production by a co-incubation of BEAS-2B cells and murine PMN is largely neutrophil NADPH oxidase-dependent

After demonstrating an important role for an intact NOX2 in the synergistic ROS production by BEAS-2B cells and an equal number of PMN, the involvement of neutrophilic NADPH oxidase in the observed synergistic IL-8 production was investigated. To this extent, we used neutrophils from WT and p47$^{phox-/-}$ mice as heat inactivation did not last longer than 1 h (data not shown).

Upon LPS treatment for either 4 (Fig. 7A) or 24 h (Fig. 7B), a significantly increased IL-8 production was observed in co-incubations of BEAS-2B and an equal number of WT PMN in comparison to the corresponding BEAS-2B mono-cultures. No immune-reactivity for human IL-8 was detected in supernatants from control and LPS-treated murine WT neutrophils, confirming that the enhanced IL-8 production in the co-incubations originates from the BEAS-2B cells. In LPS-treated co-incubations containing p47$^{phox-/-}$ PMN, IL-8 levels were significantly lower than those measured in the corresponding incubations with WT PMN, except for the cultures that were treated with 100 µg/ml LPS for only 4 h. Moreover, with the exception of the 4 h treatment with 10 µg/ml, no statistically significant difference was observed between the LPS treated co-incubations that contained p47$^{phox-/-}$ PMN and the corresponding BEAS-2B mono-cultures. Notably, after 4 h the IL-8 production induced by the highest LPS concentration (100 µg/ml) was significantly lower in all incubations compared to the corresponding incubations with the lower LPS concentration (10 µg/ml).

Interestingly, the importance of NADPH oxidase within the observed synergism was further underlined by the finding that inhibition of myeloperoxidase (MPO), another important ROS producing neutrophilic enzyme, did not influence the LPS-induced IL-8 production in the co-incubation (Fig. 7C).

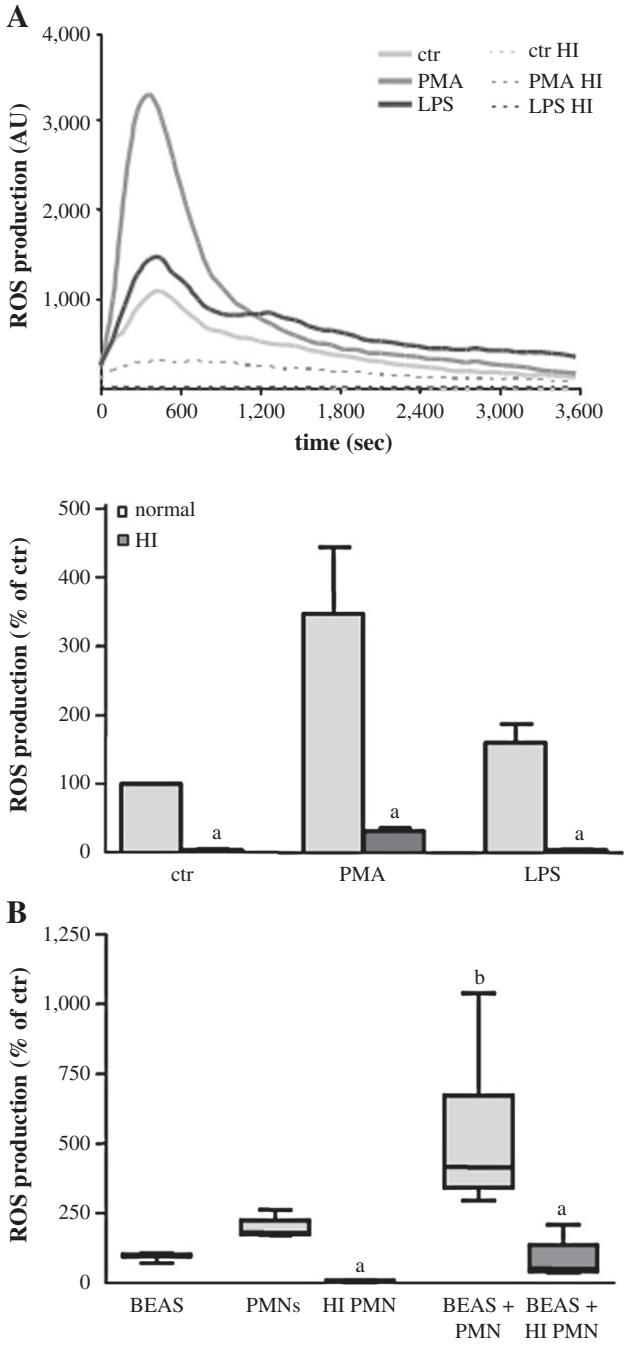

Fig. 5. Neutrophil NADPH-oxidase is implicated in the synergistic ROS-production by a PMA- or LPS-stimulated PMN-BEAS-2B (1:1) co-incubation. Upon isolation, human PMN were incubated at 46 °C for 7.5 min to induce heat inactivation (HI) of the neutrophil NADPH oxidase. Next, luminol-enhanced luminescence was used to measure the cumulative ROS production during 60 min in either PMN stimulated with PMA (100 ng/ml) or LPS (100 µg/ml) (panel A) or in BEAS-2B cells stimulated with LPS (100 µg/ml, panel B) in the absence or presence of HI PMN. Panel A shows representative chemiluminescence curves and their quantitative analyses in mean ± S.E.M. while panel B displays median, range and inter-quartile ranges of n = 6 with 3 different PMN donors. a = $P<0.05$ compared to the corresponding incubation without HI. b = $P<0.05$ compared to the corresponding incubation in only BEAS-2B cells or only PMN. c = $P = 0.062$ compared to the corresponding incubation without HI.

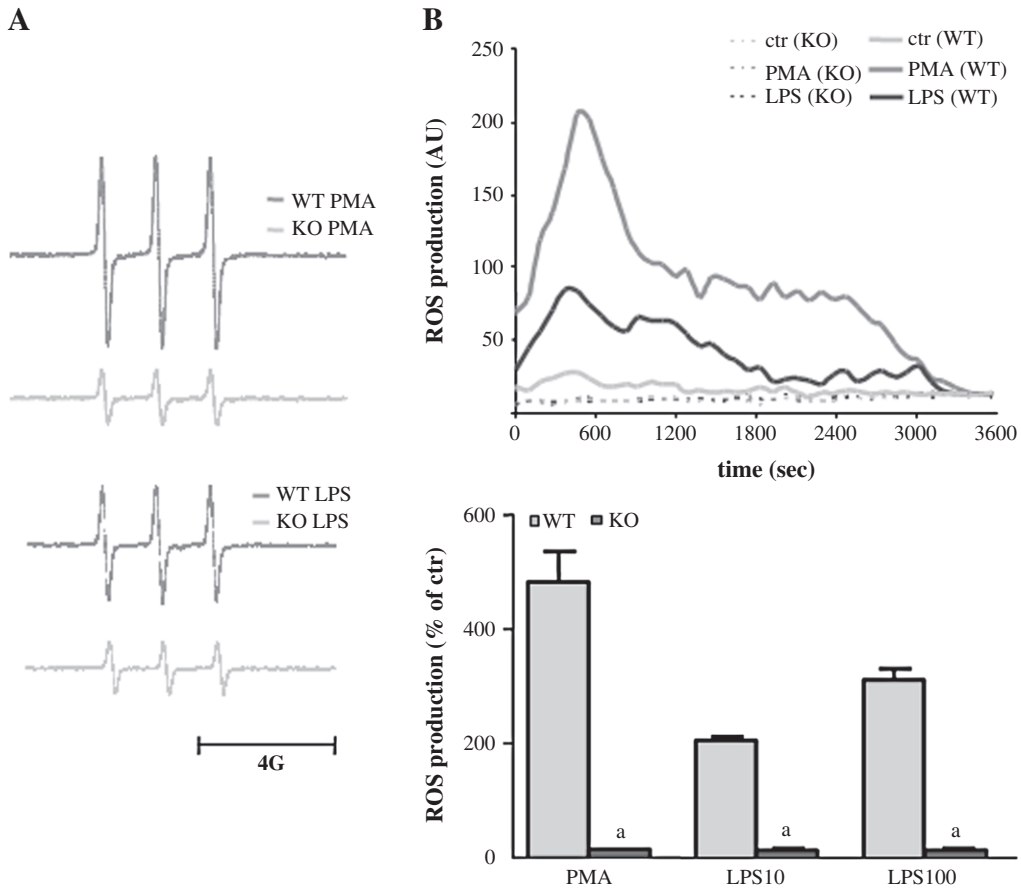

Fig. 6. Murine PMN isolated from p47$^{phox-/-}$ knockout mice are not capable of producing ROS upon stimulation with PMA or LPS. ROS production by bone-marrow derived PMN obtained from either WT or p47$^{phox-/-}$ KO mice was analyzed. Panel A shows a representative EPR spectrum of CP nitroxide resulting from the reaction of spin probe CPH with superoxide produced by murine WT or KO PMN stimulated with 50 ng/ml PMA or 10 μg/ml LPS. Panel B represents the cumulative ROS production by murine WT or KO PMN stimulated with PMA (100 ng/ml) or LPS (10 or 100 μg/ml) using luminol-enhanced luminescence during 60 min. Both representative curves and their quantitative analyses in mean ± S.E.M are displayed. a = $P<0.05$ compared to the corresponding incubation with WT PMN.

4. Discussion

Various lung diseases, including chronic obstructive pulmonary disease and cystic fibrosis, are characterized by an increased number of neutrophils [40,41]. It has been discussed that patients suffering from such diseases are more susceptible toward exogenous pro-inflammatory triggers. In order to explore possible mechanisms underlying this susceptibility issue, we have investigated the influence of neutrophils on LPS-induced pro-inflammatory signaling in BEAS-2B cells. To this end, we have applied an in vitro model based on our previous work [26], in which BEAS-2B cells and neutrophils were co-incubated to mimic possible crosstalk between these structural and inflammatory cell types. In LPS treated co-incubations a marked synergism in ROS production, release of IL-8 and neutrophil chemotactic activity could be observed. Moreover, LPS-induced phosphorylation of ERK1/2 and IκBα as well as NFκB RelA DNA binding activity was significantly augmented in the presence of neutrophils. Blocking the neutrophilic NADPH oxidase activity, either temporarily by heat inactivation or indefinite by applying the p47$^{phox-/-}$ knockout mouse model, resulted in the annulment of the observed synergistic ROS production and IL8-release. The results of these studies demonstrate that the effects of LPS toward lung epithelial cells are amplified in the presence of neutrophils.

It is known that LPS exerts its effects in epithelial cells by binding to TLR 4, a receptor that is indeed also functionally active in our BEAS-2B cells [32,36,42]. By activating TLR4, LPS induces the transcription of various pro-inflammatory cyto- and chemokines including IL-8 but also GM-CSF and MIP-3α [32,35]. Previously, we have shown that in human bronchial epithelial cells, the general response mechanism to LPS involves extracellular ATP release leading to the activation of epithelial DUOX1. Subsequently, this DUOX1-dependent H_2O_2 production was shown to induce a pro-inflammatory cascade including the activation of ERK1/2 and NF-κB and the production of IL-8 [1]. These previous observations are in concordance with our present findings, showing LPS-mediated TLR4 activation of a pro-inflammatory cascade in the BEAS-2B cells involving ROS production and resulting in increased IL-8 production.

In the presence of neutrophils, BEAS-2B cells showed a markedly enhanced LPS-triggered activation of ERK1/2 and NF-κB, accompanied by a synergistic IL-8 release. We postulate that these effects are a direct consequence of the close contact between the two cell types, leading to the concurrently observed synergistic ROS generation in this co-incubation, crucially driven by the neutrophilic NADPH oxidase NOX2 (Fig. 8). The experimental set-up comprising a co-culture model does not only closely resemble the in vivo situation in the lung, but has also been shown to be essential to induce damage to lung epithelial cells by neutrophilic ROS production. Indeed, we have previously shown that averting this close contact by using only supernatants or inserts abolishes the ROS diffusion from the neutrophils to the epithelial cells needed to induce damage in the latter [26,43].

The NOX2 enzyme complex of activated neutrophils is well known to produce large amounts of superoxide anion radical [44], which can dismutate into the more stable, membrane-diffusible ERK1/2- and

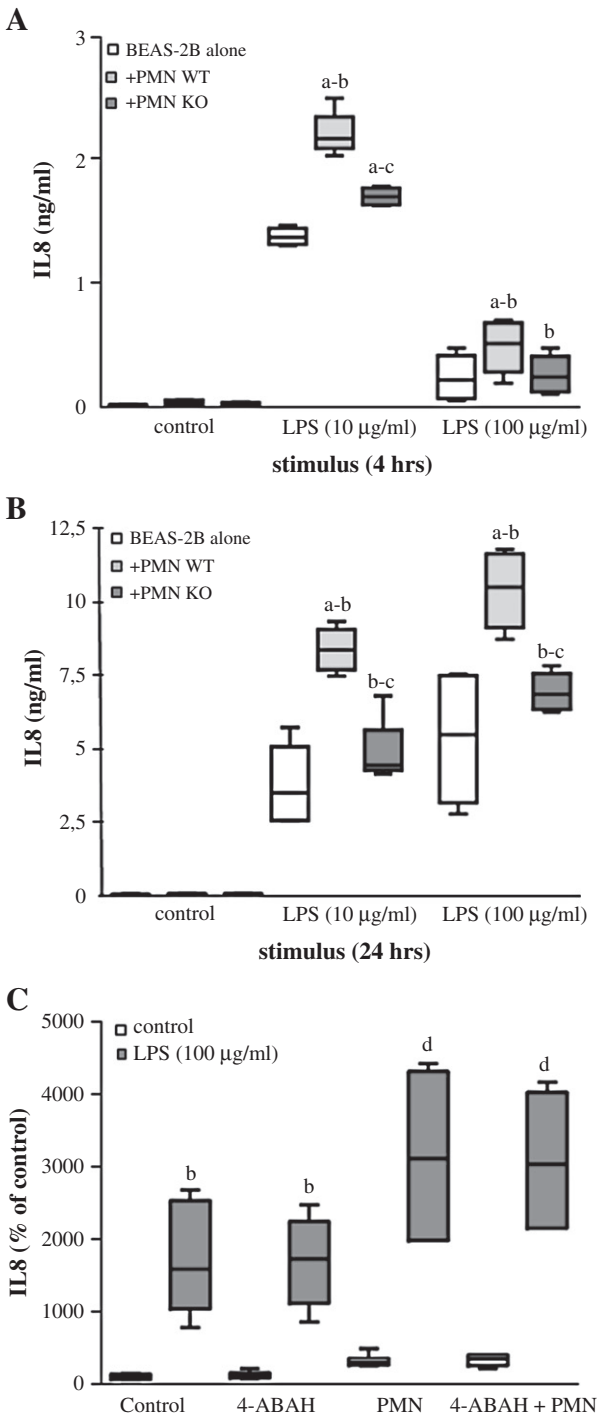

Fig. 7. Neutrophil NADPH oxidase is implicated in the synergistic IL-8 production by an LPS-stimulated co-incubation of human BEAS-2B cells and murine PMN (ratio 1:1). The IL-8 secretion was measured in the medium of starved BEAS-2B cells that were stimulated with LPS (10 or 100 µg/ml) for either 4 (panels A and C) or 24 (panel B) hours in the absence or presence of murine WT or KO PMN (1:1 ratio) or upon pre-incubation with the MPO inhibitor 4-ABAH in the absence or presence of human PMN (panel C). Data are expressed as median, range and inter-quartile ranges. a = $P<0.05$ compared to corresponding incubation with BEAS-2B only. b = $P<0.05$ compared to the corresponding incubation without LPS. c = $P<0.05$ compared to the corresponding incubation with WT PMN. d = $P<0.05$ compared to the corresponding incubation without human PMN.

NF-κB activator H_2O_2 [45–48]. Interestingly, recent findings in human lung fibroblasts indicate that superoxide itself is also capable of entering cells and activating ERK1/2 [49], hence providing an additional or alternative mode of action for ROS to affect ERK1/2 and NF-κB signaling (Fig. 8). Since LPS was less potent than PMA in inducing a respiratory burst in PMN, we suggest that the observed LPS-mediated synergistic ROS formation also involves active participation of the epithelial cells via a yet-to-be unraveled mechanism. Additionally, some residual IL-8 production could be observed in the co-incubations containing PMN with an inactive NOX2. This points to other neutrophil-derived components involved in the observed synergistic ROS-production, although their contribution seems to be limited.

A major outcome of our study was the pronounced PMN-dependent production of chemo-attractive IL-8 by the BEAS-2B cells. Indeed, IL-8 is considered to be the major neutrophil chemotactic factor in the lung [50,51] and therefore our findings indicate that the presence of a pulmonary neutrophil pool may facilitate the recruitment of additional neutrophils upon exposure to LPS. Whether IL-8 is also involved in the direct activation of neutrophils in our co-incubation, and may thus represent a pro-inflammatory amplification loop, remains open for debate. On the one hand, IL8 has been shown to prime and/or activate NOX2 [52,53]. On the other hand, the synergistic effects were also observed in co-incubations containing murine neutrophils, even though phagocytes from this species are poorly activated by human IL-8 as produced by the BEAS-2B cells.

In our present studies, neutrophils derived from the bone marrow of $p47^{phox-/-}$ mice were applied to explore the specific role of NOX2 in amplifying epithelial pro-inflammatory signaling as this enzyme is nowadays considered to be relevant in various diseases [54]. Our approach provides a model that addresses the role of NOX2 in neutrophilic inflammation, a situation known to occur in chronic pulmonary diseases including chronic obstructive pulmonary disease and cystic fibrosis. However, NOX2 knockout mice were initially developed to study its role in host defense as it is known that people lacking this enzyme develop chronic granulomatous disease, a condition characterized by increased infections and granuloma formation [55]. As such, Zhang et al. have recently shown that LPS induces a stronger inflammatory response in NOX2 deficient mice than in the WT animals [56]. At first sight, this finding seems to be in contrast to our findings, but it is important to consider some essential differences in the study objective and design. Zhang et al. have administered LPS intraperitoneally to systemically study endotoxin-induced inflammation under impaired defense conditions, whereas we studied this process in neutrophil-loaded conditions that mimic a primed inflammatory lung found in patients suffering from chronic pulmonary diseases. Considering the differences in outcome, it would be interesting to determine the pulmonary pro-inflammatory effects of LPS in wild type and NOX2 impaired mice already subjected to neutrophil infiltration, e.g. by using repeated LPS instillations. In such way, also the impact of chronic or recurring exposure to other noxae, including particulate air pollution, on the course of chronic neutrophilic lung diseases could be assessed.

As it holds true for many in vitro models, cell culture studies have some intrinsic limitations and therefore data from these types of experiments should be interpreted with care. For instance, our model does not take into account potential effects of other inflammatory or structural cell types that are thought to be relevant in pulmonary inflammation such as resident alveolar macrophages and the pulmonary endothelium. Perhaps the most important deficit in our current in vitro co-incubation model is that it does not include physiological mechanisms implicated in the resolution of neutrophilic inflammation. The specific recognition and subsequent pulmonary clearance of apoptotic neutrophils is recognized as an important anti-inflammatory feedback mechanism. Such regulatory loops are thought to be comprised in chronic inflammatory lung diseases and therefore considered to actually contribute to their pathogenesis. Indeed, current studies are undertaken to pharmacologically target these apoptosis-driven clearance pathways [57]. Another possible discrepancy between our in vitro model and the in vivo situation

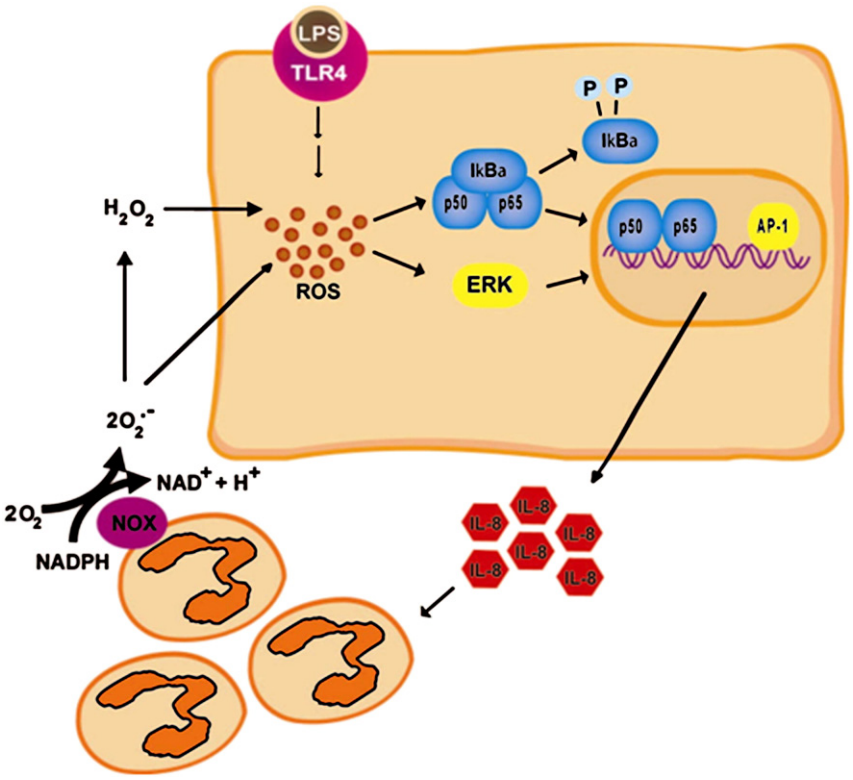

Fig. 8. Schematic representation of the interplay between pulmonary epithelial cells and neutrophils resulting in synergistic ROS production and inflammation. Upon LPS activation via binding to TLR-4, pulmonary epithelial cells will produce large amounts of ROS that can activate the redox-sensitive transcription factors ERK1/2 and NF-κB. These activated transcription factors will subsequently induce IL-8 production that can stimulate present neutrophils. Upon their stimulation, neutrophil NADPH oxidase will produce large amounts of superoxide anion radicals that will further enhance the pro-inflammatory ROS-ERK1/2-NF-κB-IL-8 loop.

might include individual differences in neutrophil serine proteases, including neutrophil elastase (NE) and protease 3 (PR3). Indeed, it has been shown that both NE and PR3 are implicated in antimicrobial defense by enhancing neutrophil-dependent inflammation and inducing IL-8 production via epithelial signaling [58,59]. Moreover, a protease-antiprotease imbalance in favor of the first has been suggested to play a role in the pathogenesis of various chronic lung diseases including COPD and asthma [60,61]. However, the possible interference of such proteases has been intercepted in our studies by i) using the same batch of donors for all different experiments or ii) applying murine neutrophils from animals with a genetically identical background except the knocking down of one subunit of NADPH oxidase, which has not been linked to other biological functions or pathways thus far. The role of these serine neutrophil proteases in the observed crosstalk between lung epithelial cells and neutrophils in vivo remains interesting and therefore worthwhile investigating.

Although the described synergism was observed in a relatively basic method to investigate the interplay between pulmonary epithelial cells and neutrophils, this model did enable us to investigate the complete interaction between both cell types as it is also occurring in vivo. The patho-physiological relevance of the performed co-incubations exceeds that of single cell type models to explore the effects of individual inflammatory and/or neutrophil-derived components such as TNF-α and H_2O_2 respectively. The relevance of our model can also be deducted from the fact that the observed synergism in IL-8 release is only partly dependent of NOX2. Moreover, the currently applied model may also be used for the screening of potential inhibitors of this synergism such as (dietary) antioxidants.

In conclusion, our findings strongly support the high impact of the interplay between neutrophils and epithelial lung cells as well as the key role of NOX-2 activity and IL-8 production in this crosstalk. Especially in patients suffering from chronic inflammatory lung diseases hallmarked by infiltrated neutrophils, recurring exposure to LPS and possibly other airborne pathogens may pose a significantly increased health hazard. Consequently, modulating the observed interplay by using natural or synthetic NOX2 inhibitors might be a successful new approach in the treatment of these diseases.

Role of the funding source

Both funding sources had no involvement in the study design; collection, analysis and interpretation of data; writing of the report and decision to submit the paper for publication.

Authorship

AWB conceived and carried out the study, analyzed the data and drafted the manuscript. KG participated in the isolation of the PMN and the performance of the co-incubations. DvB performed part of the EPR experiments. KL carried out parts of the western blot analyses. CA sacrificed the animals and prepared the femurs and tibias for the PMN isolation. RB performed the RT-PCR and MPO inhibitor experiments. GH, AB, CA, FvS and RS co-conceived the study, participated in its design and helped to analyze the data and to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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